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Invited review

Prevalence and influence of cys407* *Grm2* mutation in Hannover-derived Wistar rats: mGlu2 receptor loss links to alcohol intake, risk taking and emotional behaviour

Christian M. Wood [a, 1], Celine S. Nicolas [a, 1], Sun-Lim Choi [a], Erika Roman [b], Ingrid Nylander [b], Alberto Fernandez-Teruel [c], Kalervo Kiianmaa [d], Przemyslaw Bienkowski [e], Trynke R. de Jong [f], Giancarlo Colombo [g], Denis Chastagnier [h], Keith A. Wafford [i], Graham L. Collingridge [a], Sheryl J. Wildt [j], Becky L. Conway-Campbell [k], Emma S.J. Robinson [a], David Lodge [a, *]

[a] School of Physiology, Pharmacology and Neuroscience, University of Bristol, Bristol BS8 1TD, UK
[b] Neuropharmacology, Addiction and Behaviour, Department of Pharmaceutical Biosciences, Uppsala University, P.O. Box 591, 751 24 Uppsala, Sweden
[c] Department of Psychiatry and Forensic Medicine, Institute of Neurosciences, Autonomous University of Barcelona, Bellaterra, 08193 Barcelona, Spain
[d] Department of Alcohol, Drugs and Addiction, National Institute for Health and Welfare, POB 30 00271 Helsinki, Finland
[e] Department of Pharmacology, Institute of Psychiatry and Neurology, Warsaw, Poland
[f] Department of Behavioural and Molecular Neuroendocrinology, University of Regensburg, 93040 Regensburg, Germany
[g] Neuroscience Institute, Section of Cagliari, National Research Council of Italy, I09042 Monserrato, CA, Italy
[h] Janvier Labs, CS4105 Le Genest-Saint-Isle, F-53941 Saint-Berthevin, France
[i] Eli Lilly Research Laboratories, Windlesham, Surrey GU20 6PH, UK
[j] Envigo, 8520 Allison Pointe Boulevard, Indianapolis IN 46250, USA
[k] Henry Wellcome Laboratories for Integrative Neuroscience and Endocrinology, University of Bristol, Dorothy Hodgkin Building, Whitson Street, Bristol BS1 3NY, UK

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ABSTRACT

Modulation of metabotropic glutamate 2 (mGlu2) receptor function has huge potential for treating psychiatric and neurological diseases. Development of drugs acting on mGlu2 receptors depends on the development and use of translatable animal models of disease. We report here a stop codon mutation at cysteine 407 in *Grm2* (cys407*) that is common in some Wistar rats. Therefore, researchers in this field need to be aware of strains with this mutation. Our genotypic survey found widespread prevalence of the mutation in commercial Wistar strains, particularly those known as Han Wistar. Such Han Wistar rats are ideal for research into the separate roles of mGlu2 and mGlu3 receptors in CNS function. Previous investigations, unknowingly using such mGlu2 receptor-lacking rats, provide insights into the role of mGlu2 receptors in behaviour. The *Grm2* mutant rats, which dominate some selectively bred lines, display characteristics of altered emotionality, impulsivity and risk-related behaviours and increased voluntary alcohol intake compared with their mGlu2 receptor-competent counterparts. In addition, the data further emphasize the potential therapeutic role of mGlu2 receptors in psychiatric and neurological disease, and indicate novel methods of studying the role of mGlu2 and mGlu3 receptors.

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Abbreviations: mGlu2 receptor, metabotropic glutamate receptor 2; RGD, Rat Genomic Database.

* Corresponding author.
E-mail address: david.lodge@bristol.ac.uk (D. Lodge).

[1] These two authors made equal contributions.

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Contents

1. Introduction

The metabotropic glutamate 2 (mGlu2) receptor belongs to the family of G-protein coupled glutamate receptors that modulate transmission at synapses throughout the mammalian central nervous system, and that have been proposed as major targets for the development of drugs for human psychiatric and neurological diseases (Niswender and Conn, 2010; Nicoletti et al., 2011; Chaki et al., 2013; Li et al., 2015). The mGlu2 receptors signal through $G\alpha_{i/o}$ proteins inhibiting adenyl cyclase and reducing cAMP (Tanabe et al., 1992), cascading into effects on multiple systems including PKA/MAPK, GSK-3β, Src kinase, AMPA and NMDA receptors etc (Pin and Duvoisin, 1995; Harris et al., 2004; Trepanier et al., 2013; Wang et al., 2013). They also signal through Gβ/γ inhibiting calcium channels (Chavis et al., 1994; Scanziani et al., 1995) and activating potassium channels (Knoflach and Kemp, 1998; Chavez-Noriega et al., 2002). The major established physiological function of mGlu2 receptors is to modulate synaptic transmission as presynaptic auto- and hetero-receptors at glutamatergic and GABA-ergic terminals (Battaglia et al., 1997; Cartmell and Schoepp, 2000; Smolders et al., 2004). The perisynaptic location of mGlu2 receptors ideally positions them for sensing glutamate overflow (Petralia et al., 1996; Shigemoto et al., 1997) and release from astrocytes (Moran et al., 2005; Kalivas, 2009).

Such complexity of the actions of a single receptor subtype confounds attempts at predicting effects of exogenous agonists or antagonists of mGlu2 receptor on whole animal behaviours and hence their therapeutic potential. Nevertheless the predicted potential for mGlu2/3 receptor agonists based on limiting glutamate release has been borne out in animal models of schizophrenia (Schoepp and Marek, 2002), anxiety (Helton et al., 1998; Swanson et al., 2005), cerebral ischaemia (Bruno et al., 2001), epilepsy (Smolders et al., 2004), drug addiction (Kalivas, 2009) and chronic pain (Chiechio et al., 2010). There has also been some limited success with clinical studies (Grillon et al., 2003; Patil et al., 2007; Dunayevich et al., 2008) but this has not yet led to an approved drug. Clearly, the importance of understanding the role of mGlu2 receptors in physiology and pathology cannot be overstated.

One of the issues has been that orthosteric agonists and antagonists do not separate between mGlu2 and mGlu3 receptors (Nicoletti et al., 2011), which have different, and possibly opposing effects (Corti et al., 2007). To overcome this problem we recently used a new selective mGlu2 receptor agonist, LY395756, and its active enantiomer, LY541850 (Dominguez et al., 2005), to separate between the roles of mGlu2 and mGlu3 receptors in synaptic events (Ceolin et al., 2011; Hanna et al., 2013). However we found that many of the outbred Wistar rats studied were unresponsive to the selective mGlu2 agonist; this apparent anomaly was traced using Western blotting to the lack of mGlu2 receptor expression in some Wistar rats (Ceolin et al., 2011). Such animals being used for animal modeling of human diseases clearly produce misleading results when studying the roles of mGlu2 receptors. For example, mGlu2/3 agonists, known to reduce the phencyclidine-induced hyperlocomotion in other rat strains (Moghaddam and Adams, 1998; Cartmell et al., 2000; Monn et al., 2007), do not show this effect in Wistar rats lacking mGlu2 receptors (Wood et al., 2014).

Because of the demonstrated potential of the mGlu2 receptor as a therapeutic target, this finding is of critical importance to the research community. Immediately questions arise as to i) why is the mGlu2 receptor missing from some rats and ii) how frequently does this occur in populations of rats used in laboratory studies. We report here the occurrence of a single point mutation in exon 3 of the *Grm2* gene, which results in a premature stop codon at cysteine 407 of the mGlu2 receptor, and resultant loss of functional protein expression. We also report the high frequency of this mutant genotype in certain outbred and inbred rat lines that are commercially available or selectively bred, and we discuss its influence on behavioural characteristics.

2. Methods

2.1. Animals

For the initial studies, Wistar rats from Banting & Kingman Ltd.

(UK), Harlan Laboratories (UK) and Charles River Laboratories (UK), were housed in pairs under temperature controlled conditions in standard laboratory housing. Experiments were conducted in accordance with the Animals (Scientific Procedures) Act 1986 and approved by local ethical review (University of Bristol). Sources, and derivation details where appropriate, of animals used in the genotyping survey are given in the Results section and in Tables 1–3.

2.2. Initial investigation

Briefly, spare hippocampal slices from rats determined electrophysiologically to be sensitive or insensitive to a selective mGlu2 receptor agonist (see Mercier et al. (2013) for Methods) were frozen at −80 °C and RNA subsequently extracted using Qiagen RNeasy kit according to the manufacturer's protocol. A first strand synthesis was performed using SuperScript III First Strand Synthesis Supermix (ThermoFisher Scientific) with Oligo(dT) according to the manufacturer's protocol. cDNA were then kept at −20 °C before PCR amplification. Initially, four pairs (A-D) of custom DNA oligonucleotides were originally designed to cover most of *Grm*2 mRNA (NM 001105711.1), namely nucleotides 131-147 and 854-834 (A), 819-838 and 1430-1411 (B), 1419-1439 and 2166-2147 (C) and 2003-2023 and 2824-2805 (D). A fifth pair of primers, 1142-1161 and 1733-1714 (E) was subsequently ordered. All PCR primers were purchased from Sigma Aldrich (UK) and used to amplify the appropriate stretches of hippocampal cDNA. Following 35 PCR cycles and confirmation of correct PCR amplification by gel electrophoresis, the amplified cDNA was purified, prepared and sent to Source Bioscience (UK) for Sanger sequencing with the same primers. The sequencing data were analysed using CodonCode Aligner (v5.1.5) and expressed as chromatograms for illustration (Fig. 1B).

2.3. Subsequent genotyping

Following the initial discovery of the cys407* mutation, the protocol for genotyping was refined and focused on the stretch of gDNA, containing the mutation, using the following primers: nucleotides 1301-1320 and 1488-1469 (NM 001105711.1) included in exon 3. With this PCR primer pair (F), the method described above was used for genotyping tissue in a survey of other outbred and inbred rat strains as indicated in Tables 1 and 2. To collect tissue samples, rats were euthanized by equivalent of UK Schedule 1 methods or, where appropriate, gently restrained to collect ear or tail tissue. Tissue was frozen at −80 °C, packaged in dry ice and, as necessary, shipped to the University of Bristol for gDNA preparation and assaying as above, with gDNA extracted using Qiagen DNeasy kit according to the manufacturer's protocol.

2.4. Allelic discrimination

In parallel with the above genotyping, allelic discrimination was used to detect the presence or absence of the same mutation in several strains from Harlan (now Envigo) Laboratories, Indianapolis (see Table 1 for details of rat lines examined). All animal tissue collection protocols used in this study were approved by the Envigo IACUC. Ear pinnae of approximately 2 mm were collected from each animal tested and shipped frozen overnight to the Envigo genetic testing services laboratory, located at the Bionomics and Research Technology Center (BRTC) in Piscataway, New Jersey. SNP genotypes were determined using Taqman chemistry with probes and primers designed using Primer Express v.3.0. Primer sequences include: Forward Primer: TGCCCTCTGTCCCAACAC; Reverse Primer: GCGGCGCCCATTGAC; Reporter 1: TAGCATCGCAGAGGTG; Reporter 2: CATAGCATCTCAGAGGTG. Specific PCR cycling conditions are as follows: 95 °C, 10 min; (95 °C, 30 s; 60 °C, 1 min) x 40. Data were collected upon completion of PCR amplification in an end plate read protocol and were analysed using ABI Fluidigm ViiA7 Real-Time PCR System.

2.5. Western blot

To assess the endogenous expression of mGlu2 receptor protein in mutant and wild-type rats, western blotting was performed with mGlu2 KO mouse tissue as a negative control (Fig. 1C). Cortical tissues were lysed by RIPA buffer (50 mM Tris-HCl (pH 6.8), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% NP-40, 0.5% SDS, 0.25% Na-deoxycholate, 1 × protease & phosphatase inhibitor cocktail (Cell Signaling)) and immobilized on the PVDF membrane (Millipore, IPVH00100). The mGlu2 receptor antibody (Millipore, 07-261-I, 1:1000) and alpha-tubulin antibody (Cell Signaling, #3873, 1:10,000) were used as primary antibodies.

2.6. Rat Genome Database (RGD) analysis

Prevalence analysis of the cys407* mutation was conducted within rat strains contained in the RGD. Rat strains containing variant information were analysed using the Variant Visualizer tool (http://rgd.mcw.edu/rgdweb/front/config.html) and the Genome Database browser (http://rgd.mcw.edu/jbrowse). Observation of nonsense mutations within the *Grm*2 gene (Rn5; RGSC Rnor_5.0) at chromosome 8, nucleotide 114,712,836 are reported.

3. Results

3.1. Discovery of the cys407* mutation in Grm2

Hippocampal cDNA from 3 B&K Wistars (Bkl:WI), 'insensitive' to the selective mGlu2 receptor agonist, LY541850, defined electrophysiologically (Mercier et al., 2013), and 1 'sensitive' control Charles River Wistar (Crl:WI) was sequenced using the 4 pairs of initial primers (A-D). These provided nucleotide sequencing from nt 141-843 (A), 829-1417 (B), 1432-2153 (C) and 2013-2814 (D) and indicated no mutations within the *Grm2* cDNA. The small uncovered stretch of DNA from 1417 to 1432 was subsequently sequenced using primer pair E and the resultant Sanger chromatograms for all 3 'insensitive' Wistar rats showed a single point mutation at nt1419 in exon 3; a cytosine was replaced by an adenine resulting in a stop codon (TGA) rather than the codon (TGC) for cysteine at amino acid 407 of the mGlu2 receptor protein (Fig. 1). No other mutations within the *Grm2* gene were observed in any of the 4 rats. The presence of the cys407* mutation was subsequently confirmed with the primer pair F using other tissue samples, defined electrophysiogically with LY541850 (Mercier et al., 2013). Such a premature stop codon provides the explanation for the absence of protein as confirmed by Western blotting (Fig. 1C) and hence the lack of effect of a selective mGlu2 receptor agonist in some Wistar rats (Ceolin et al., 2011). This mutation is the same as reported independently in the Wistar-derived alcohol-preferring P rats (Zhou et al., 2013).

3.2. Prevalence of the mutation in commercially available Wistar rats

To assist in the assessment of the frequency of the *Grm2* cys407* mutation in commercially available stocks of Wistar rats, tissue samples from laboratory animal suppliers were genotyped. We initially found that 100% of B&K Wistars (Bkl:WI) and Harlan HSD Han Wistars (HsdHan:WIST) were 100% homozygous mutants

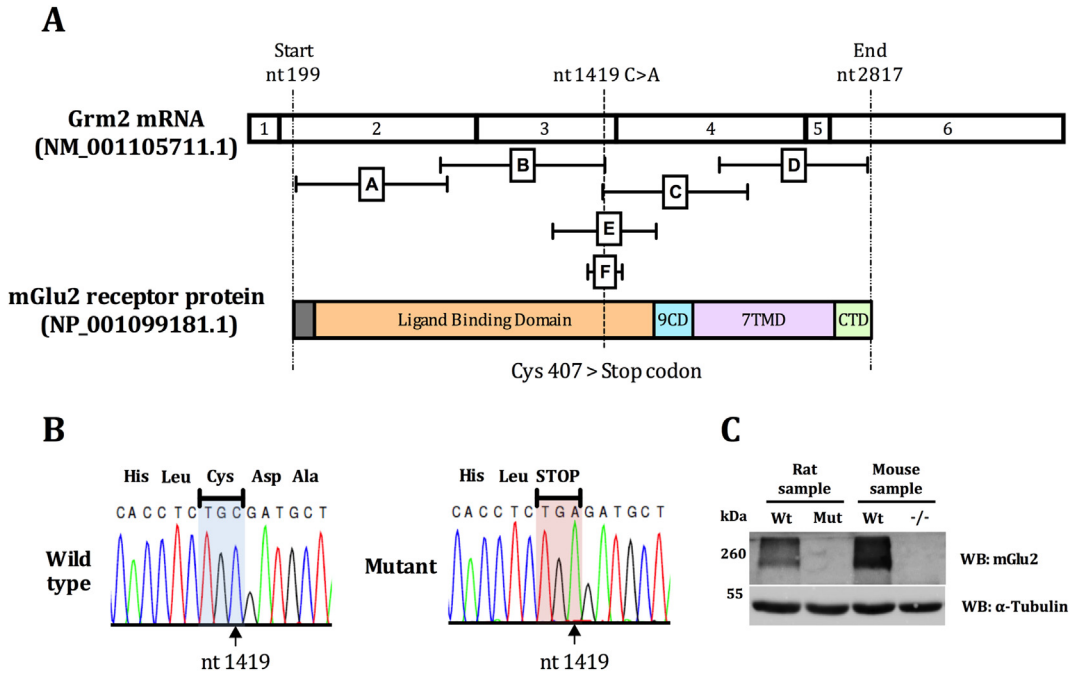

Fig. 1. Discovery of the cys407* mutation within the *Grm2* gene and loss of mGlu2 receptor expression in Han Wistar rats. (A) Structure of mGlu2 receptor cDNA showing the regions mapped by five specific primers (A-E) used for Sanger sequencing. The site of the nonsense mutation at nt1419, results in a stop codon at cysteine 407, and is indicated on both the cDNA and protein. (B) Typical *Grm2* wild type (TGC) and mutant (TGA) chromatograms from Sanger sequencing; arrows indicating the mutation site at nt1419. (C) Western blots indicating the loss of mGlu2 receptor protein in homozygous cys407* mutant rats (Mut); the blots from mGlu2$^{-/-}$ mice and wild type mice demonstrate the specificity of the antibody. α-tubulin was used as a loading control.

whereas 100% of Charles River Wistars (Crl:WI) were 100% homozygous wild-type (Table 1). The survey was extended to other Wistar strains in Europe, the United States and Israel. A simple scan of the data in Table 1 shows that Han Wistars, including RCC Han Wistars (HsdRcc:WIST), are largely homozygous or heterozygous mutants whereas Wistars without the 'Han' designation are

Table 1
In-house genotypic analysis for the *Grm2* cys407* allele in rat lines from commercial suppliers. Genotyping was conducted using Sanger sequencing or allelic discrimination, with the latter indicated by [1]. Allelic frequency denotes frequency of the mutant cys407* allele within each sample. Han Wistar sources used in the Palm et al., (2011a,b) studies are indicated by [2]. Furthermore, the following 14 inbred strains from Harlan Labs (USA) were analysed by allelic discrimination and all were homozygous wild type: ACI/Seg, LEW/SsNHsd, F344/NHsd, WKY/NHsd, BN/SsNHsd, LE/CpbHsd, SHR/NHsd, SS/JrHsd, SR/JrHsd, LEW/HanHsd, BN/RijHsd, F344/NHsd, WF/NHsd (n = 5 per strain) and HsdCpb:WU (n = 80).

Commercial supplier	Common name	Nomenclature	N	Genotypic outcome	Allelic frequency
Banting & Kingman (UK)	B&K Wistar	Bkl:WI[2]	14	100% mutant	1.0
Scanbur (Sweden)	Scanbur B&K Wistar	Sca:WI	9	45% mutant 33% hetero 22% wild type	0.61
Harlan Labs. (UK)	HSD Han Wistar	HsdHan:WIST	50	100% mutant	1.0
Harlan Labs. (UK)	RCC Han Wistar	HsdRcc:WIST	6	83% mutant 17% hetero	0.92
Harlan Labs. (USA)[1]	HSD Han Wistar	HsdHan:WIST	50	100% mutant	1.0
Harlan Labs. (USA)[1]	RCC Han Wistar	HsdRcc:WIST	50	98% mutant 2% hetero	0.99
Harlan Labs (Israel)	HSD Han Wistar	HsdHan:WIST	18	100% mutant	1.0
Harlan Labs (Netherlands)	RCC Han Wistar	HsdRcc:WIST[2]	6	67% mutant 33% hetero	0.83
Harlan Labs (USA)	Harlan Wistar	Hsd:WI	48	100% wild type	0.0
Harlan Labs (Israel)	Harlan Wistar	HsdOla:WI	48	100% mutant	1.0
Harlan Labs (USA)[1]	Sprague Dawley	Hsd:Sprague Dawley	96	100% wild type	0.0
Harlan Labs (USA)[1]	Dark Agouti	DA/OlaHsd	32	100% mutant	1.0
Charles River (UK)	CR Wistar	Crl:WI	50	100% wild type	0.0
Charles River (Germany)	CR Wistar	Crl:WI	10	50% wild type 50% hetero	0.25
Charles River (UK)	CR Han Wistar	Crl:WI(Han)	20	60% hetero 35% mutant 5% wild type	0.48
Charles River (UK)	Wistar Kyoto	WKY/NCrl	5	100% wild type	0.0
Charles River (France)	CR Han Wistar	Crl:WI(Han)	20	40% mutant 55% hetero 5% wild type	0.48
Taconic Farms (Denmark)	Taconic Han Wistar	HanTac:WH[2]	15	73% mutant 27% hetero	0.87
Janvier (France)	Janvier Han Wistar	RjHan:WI	10	60% hetero 40% wild type	0.3
Janvier (France)	Janvier Dark Agouti	DA/HanRj	10	100% mutant	1.0

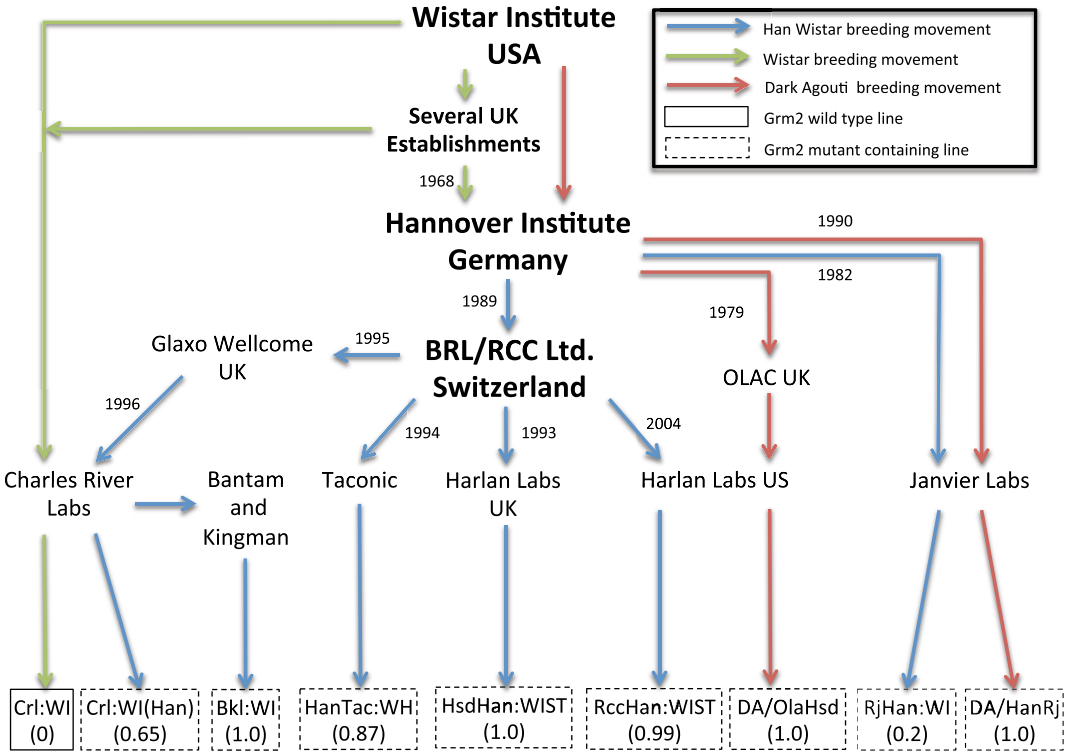

Fig. 2. Dendrogram showing simplified scheme of derivation of commercial strains of Wistar, Han Wistar and Dark Agouti rats. For simplification, the complex pathway between the Wistar and Hannover Institutes, which included Glaxo (UK) Ltd, ICI (UK) Ltd and Allington Farms, Porton Down is abbreviated to 'Several UK Establishments'. Modified from IFFA Credo Ltd. scheme (Charles River Ltd.). The figures in parenthesis represent the allelic frequency for each commercial strain.

primarily wild-type, with the apparent exceptions of Harlan HsdOla Wistars (HsdOla:WI) and the two B&K Wistar lines (Table 1). The B&K Wistars are, however, known to be derived via the Hannover Institute (Ceolin et al., 2011; Palm et al., 2011a, Fig. 2) and hence these lines can be classified as Han Wistars.

Two non-Wistar lines in our survey of commercial sub-strains showed the cys407* mutation, namely the Dark Agouti lines, DA/OlaHsd and DA/HanRj (Table 1). A list of other inbred commercial lines that did not carry the mutation is presented in the legend to Table 1.

3.3. Prevalence of the mutation in rat lines selectively bred for particular behavioural characteristics

Because some commercial outbred Wistars lacking expression of mGlu2 receptors (Table 1) have been reported to have an anxiety-like phenotype (Ceolin et al., 2011) and an increased voluntary alcohol intake and risk–related behaviours (Palm et al., 2011a, 2011b; Momeni et al., 2015), we extended the investigation of the cys407* mutation prevalence to small cohorts from lines selectively bred for particular behavioural phenotypes (Table 2).

Table 2
Prevalence analysis for the *Grm2* cys407* allele in rat lines selectively bred for particular behavioural characteristics. Genotypic outcome and cys407* mutant allele frequency have been calculated for each rat line. Sources of the tissue for in-house genotyping are shown, with previously published genotyping data indicated by #.

Common name	Nomenclature	Source	N	Genotypic outcome	Allelic frequency
Roman High Avoidance	RHA-I	Autonoma University, Barcelona, Spain	6	100% mutant	1.0
Roman Low Avoidance	RLA-I		6	100% wild type	0.0
Alko Alcohol-Preferring	AA	National Institute for Health and Welfare, Helsinki, Finland	12	92% wild type 8% hetero.	0.04
Alko Non Alcohol-Preferring	ANA		12	100% wild type	0.0
General Absence Epilepsy	GAERS	Grenoble Institut des Neurosciences, France	6	100% wild type	0.0
Non-Epileptic Control	NEC		6	100% wild type	0.0
Alcohol Preferring#	P	Zhou et al. (2013)#	125	100% mutant	1.0
Alcohol Non-Preferring#	NP	NIAAA, NIH	59	100% wild type	0.0
Warsaw Alcohol High-Preferring	WHP	Institute of Psychiatry and Neurology, Warsaw, Poland	5	60% hetero. 20% mutant 20% wild type	0.5
Warsaw Alcohol Low-Preferring	WLP		5	80% hetero. 20% wild type	0.4
Sardinian Alcohol Preferring	sP	University of Cagliari, Sardinia	10	50% hetero. 40% mutant 10% wild type	0.65
Sardinian Alcohol Non-Preferring	sNP		10	100% wild type	0.0
High Anxiety-related Behaviour	HAB	University of Regensburg, Germany	8	100% mutant	1.0
Low Anxiety-related Behaviour	LAB		9	100% mutant	1.0

The Wistar-derived Roman High- (RHA) and Low-Avoidance (RLA) rat lines were initially selected and outbred in Rome on the basis, respectively, of their good or poor acquisition of the two-way active avoidance response (Bignami, 1965) and transferred to Zürich in 1972 (Driscoll and Bättig, 1982; Driscoll et al., 1998; Steimer and Driscoll, 2003). From these RHA/Verh and RLA/Verh lines, two inbred lines (RHA-I and RLA-I) were derived and maintained at the Autonomous University of Barcelona since 1997 (Escorihuela et al., 1999; Driscoll et al., 2009). Analysis of samples from 6 RHA-I and 6 RLA-I male rats showed that all RHA-I rats and no RLA-I rats from Barcelona expressed the mutation (Table 2).

The Alko Alcohol-preferring (AA) and Alko Non Alcohol-preferring (ANA) rats were derived from a Wistar-Sprague Dawley cross in the 1960s (Eriksson, 1968). Other non-Wistar strains were bred into them for further selective breeding for alcohol preference (Hyytia et al., 1987; Sommer et al., 2006). On genotyping the AA and ANA rats (n = 12/strain), all were wild-type, except for one AA rat, which was heterozygous for the cys407* allele (Table 2).

Generalized Absence Epilepsy Rats from Strasbourg (GAERS) were selectively bred for spontaneous absence seizures from a 20-year old in-house Wistar stock in the early 1980s (Vergnes et al., 1982; Marescaux and Vergnes, 1995; Danober et al., 1998). Neither the GAERS samples (n = 6) nor the non-epileptic control rats (NEC; n = 6) contained the cys407* mutation (Table 2).

In the early 1990s in Munich, Wistar rats from Charles River (Germany) were selectively bred, based on their behaviour in the elevated plus-maze into two lines, one with High Anxiety-related Behaviours (HAB) and the other with Low Anxiety-related Behaviours (LAB; Liebsch et al., 1998). The initial HAB and LAB lines were crossed with other Wistar (Wis/Prob) rats selectively bred in Leipzig for low and high performance respectively in a shock–motivated brightness discrimination task (Hess et al., 1992). The resulting HAB and LAB breeding lines, currently maintained at the University of Regensburg, show many signs of clinical anxiety as well as abnormal aggressive behaviour (Landgraf and Wigger, 2002; Neumann et al., 2010). When genotyped for the *Grm2* mutation, all HAB (n = 8) and LAB (n = 7) rats, verified individually for their respective anxiety-like characteristics using the elevated plus maze, were homozygous for the cys407* mutation (Table 2).

The Sardinian alcohol-Preferring (sP) and alcohol Non-Preferring (sNP) lines were developed through a selective outbreeding program starting from a stock of Wistar rats bred at Morini, San Polo d'Enza, Italy (see Colombo et al., 2006). The sP rats display increased anxiety-related behaviours relative to sNP rats (Colombo et al., 1995; Roman and Colombo, 2009). When genotyped, the sP (n = 10) and sNP (n = 10) rats were clearly distinguished between lines with *Grm2* cys407* alleles only in the alcohol-preferring line, with no mutants in the sNP line (Table 2).

Similarly the selective breeding of Warsaw High alcohol-Preferring (WHP) and Low alcohol-Preferring (WLP) rats was commenced in the early 1990s from Wistar stock (Bisaga and Kostowski, 1993; Dyr and Kostowski, 2004). The WHP rats display lower anxiety-related and depressive-like behaviours than the WLP rats (Acewicz et al., 2014). Unlike many of the above lines selectively bred for alcohol intake, the WHP rats (n = 5) and WLP rats (n = 5) were similar in *Grm2* genotype, with both lines showing a mixture of *Grm2* mutants and wild types (Table 2).

Examination of the Rat Genome Database (RGD) for the cys407* mutation revealed this mutation in a number of inbred lines (Table 3), including another Dark Agouti line (DA/BklArbNsi) and the Maudsley Reactive line (MR/N; Broadhurst 1975; Blizard et al., 2015). These MR/N rats were bred from Wistars on the basis of rates of defaecation in an open field setting, the inbred strain in the RGD being homozygous for the *Grm2* mutation. On this database of 43 inbred strains, there were 9 rat lines expressing the mutation. Four of these mutant lines, BUF/N, M520, WN/N and MR/N itself, were used to derive the N/NIH heterogeneous stock rats from a total of 8 lines (Hansen and Spuhler, 1984).

Table 3
Results showing rat lines that contain the *Grm2* cys407* allele from the Rat Genome Database (RGD) using the Variant Visualizer tool (http://rgd.mcw.edu/rgdweb/front/config.html). All lines listed are homozygous for the mutant Grm2 allele. The code and RGD ID for each rat line are indicated. The variant data resources include The Royal Netherlands Academy of Arts and Sciences (KNAW), Medical College of Wisconsin (MCW), National Institute of Health (NIH) and Atanur et al. (2013). * Indicates the 4 rat lines of the 8 used to breed the N/NIH heterogeneous stock rats.

Common name	Nomenclature	RGD ID	Data resource	Variant ID
Biobreeding rats	BBDP/WorN	1598798	KNAW	588304182
Buffalo rats	BUF/N*	60986	KNAW	591803679
Dark Agouti rats	DA/BklArbNsi	1600340	KNAW	595199597
Marshall 520 rats	M520/N*	10024	KNAW	631632754
Maudsley Reactive rats	MR/N*	70449	KNAW	565287345
Milan Normotensive rats	MNS/Gib	10028	KNAW	634892321
Sabra Hypotension resistant	SBN	68130	MCW	532608734
Sabra Hypotension resistant	SBN/Ygl	631573	KNAW	641705586
Inbred Wistar NIH	WN/N*	10045	NIH	548665674

4. Discussion

Our independent finding of the cys407* mutation in *Grm2* (reported here), the description of the cys407* mutation in alcohol-preferring P rats (Zhou et al., 2013), and the low mGlu2 receptor expression in inbred Roman High Avoidance (RHA-I) Wistar-derived rats (Klein et al., 2014) demanded a widespread survey for this *Grm2* mutation among Wistar rats. This has led us to discover a preponderance of the cys407* mutation in some commercial Wistar rats and in some selectively bred lines of Wistar origin (Tables 1–3). The discussion will focus on the discovery, prevalence and origin of the mutant genotype and the implications for use in neuroscience research. We also consider how this mutation may relate to a specific behavioural phenotype.

4.1. Discovery, prevalence and origin of the cys407* mutation

In addition to our own observation that some strains of Wistar rats have reduced expression of the mGlu2 receptor (Ceolin et al., 2011), others have made similar observations, noting only silent mutations, reductions in transcript level and potential epigenetic changes (Lindemann et al., 2006; Klein et al., 2014). The molecular basis for the reduced mGlu2 receptor expression was not determined until our discovery (April 2013) of the cys407* mutation and, independently reported by Zhou et al. (2013) in the Wistar-derived P rats, a line selectively bred for alcohol consumption and

preference (Lumeng et al., 1977; Li et al., 1979). Building on this work, we have now surveyed a number of commercially available Wistar stocks including those used for behavioural profiling and assessment of voluntary alcohol intake (Palm et al., 2011a,b Momeni et al., 2015) and a few selectively-bred lines showing phenotypes that tentatively might be linked to loss of mGlu2 receptors. Lastly, we have interrogated the Rat Genome Database to further explore the prevalence of the *Grm2* cys407* mutation.

Our data show widespread distribution of the cys407* mutation in commercially available Wistar rats of different origin and from different suppliers (Table 1), particularly those with known historical derivation from the Hannover Institute (Zentral Institut fur Versuchstierzucht; Fig. 2). This may provide a clue to the origin of the mutation. Founders from the Hannover Institute were distributed to *inter alia* IFFA-Credo (later Charles River, France), Biomedical Research Laboratories (BRL; later Research Consulting Company, RCC; Switzerland) and Bury Green Farm (Glaxo, UK). These were the sources, which have given rise to commercial Han Wistar colonies expressing the cys407* mutation (Fig. 2). It can therefore be assumed that the mutation was common within the Wistar stock at the Hannover Institute. At first sight, it seems likely that the initial spontaneous mutation occurred at the Hannover Institute presumably in a single animal. An earlier event, e.g. at an establishment in the UK or even at the Wistar Institute itself (see Fig. 2) is, however, perhaps more likely; a small number of resultant mutants then being chosen by chance to act as founders at the Hannover Institute. Similarly, although Wistar rats from commercial stocks not originating via the Hannover Institute generally do not appear to contain the mutation (see Fig. 2; Tables 1 and 2), presence of the mutation in the Harlan Wistar (HsdOla:WI) and in some older inbred lines in the Rat Genome Database, including the Maudsley Reactive line (MR/N; Table 3) also suggests a pre-Hannover event. The non-Wistar Dark Agouti rats (DA/OlaHsd, DA/HanRj and DA/BklArbNsi) were surprisingly all homozygous cys407* mutants. Unfortunately, tracing the precise origins of many of these older lines is virtually impossible and so a Han Wistar lineage could not be determined, e.g. (http://www.informatics.jax.org/external/festing/rat/docs/DA.shtml).

Selective breeding for a particular behavioural trait from stock containing some mutants may lead to a change in frequency of the 407* mutation, if this allele influences the sought for phenotype (see below). Why, however, did loss of the mGlu2 receptor result in such rats becoming the most numerous genotype in some commercial colonies? Was it just by chance that the rats used as founders or for revitalizing some colonies were mostly *Grm2* mutant rats or does the mutation provide some phenotypic advantage in commercial breeding laboratories? Obvious explanations might be that cys407* mutant rats are more efficient in terms of growth or fecundity. Something as simple as handling characteristics or interaction with humans may also affect choice of individual rats for breeding in establishments where no phenotype is actively sought. Further behavioural studies comparing 'Han' and 'non-Han' lines of Wistar rats (e.g. Palm et al., 2011a) may eventually disclose some as yet unknown characteristic, which leads to *Grm2* mutant individuals being chosen for breeding.

In addition to evidence of a prevalence of the mutation in outbred stocks of Wistar rats of Han origin, we have also shown *Grm2* genotypic heterogeneity between certain selectively bred lines. This raises the possibility that the presence of specific behavioural characteristic, e.g. alcohol preference, may be linked to the expression of mGlu2 receptors and thus selection of animals for such behaviours will result in lines with different prevalence of the mutation. In particular, the link between the *Grm2* mutation, alcohol intake, anxiety-related and risk-related behaviours are of particular interest and are discussed in more detail below.

4.2. Behavioural characteristics of sub-strains lacking mGlu2 receptors

In addition to the electrophysiological differences noted with selective mGlu2 receptor agonists (Ceolin et al., 2011; Hanna et al., 2013; Lucas et al., 2013; Mercier et al., 2013; Sanger et al., 2013), differences in behaviours related to emotionality were also observed (Bert et al., 2001; Ceolin et al., 2011; Palm et al., 2011a; Honndorf et al., 2011). When tested in the multivariate concentric square field™ test (MCSF), the main segregating factors for the Han Wistar rats were lower general activity but increased activity in areas associated with risk, i.e. higher risk taking behaviour (Palm et al., 2011a). With the knowledge of distribution of the cys407* mutation in the rat strains from Table 1, this behavioural correlation with genotype can be illustrated (Fig. 3). Whilst this provides an interesting insight into the potential link between the mutation and behavioural characteristics, it should be noted that most studies have only examined a small number of strains and there is considerable behavioural variation depending on the task used and comparison groups (Palm et al., 2011a; Goepfrich et al., 2013; Momeni et al., 2015).

Among lines selected for behavioural phenotype, the RHA-I rats (homozygous for the cys407* mutation) were originally selected for breeding based on rapid acquisition of avoidance responses in shuttle boxes (Bignami, 1965; Driscoll and Bättig, 1982; Driscoll et al., 1998; Escorihuela et al., 1999). The fact that only 5 generations were needed to establish this phenotype (Bignami, 1965) suggests relatively high penetrance of the genotype. The RHA-I rats show aspects of impulsivity, risk-taking and sensation seeking (Escorihuela et al., 1999; Lopez-Aumatell et al., 2009a; Moreno et al., 2010; Klein et al., 2014), characteristics which have some parallels with the behavioural phenotype reported in commercially available Han Wistars (Palm et al., 2011a; Momeni et al., 2015).

Whilst the distribution of the *Grm2* mutation within different outbred Wistar populations cannot be specifically linked to emotional behaviour, there is some evidence to suggest anxiety-related behaviour in mutant versus non-mutant animals (Ceolin et al., 2011) or risk/impulsivity-related behaviours (Palm et al., 2011a; Klein et al., 2014). However, whilst the *Grm2* mutation will have particular influences on behaviour, differences in the methods used to study these behaviours (Steimer and Driscoll, 2003; Diaz-Moran et al., 2012; Klein et al., 2014) and other genetic and environmental/experimental features will overlay and complicate interpretation.

4.3. Alcohol intake in sub-strains lacking mGlu2 receptors

Wistar rats from commercial sub-strains lacking the mGlu2 receptor tend to consume more alcohol than non-Han Wistar rats (Palm et al., 2011b). However, there are inconsistencies in the data relative to our analysis of the prevalence of the mutation. Low voluntary alcohol intake was observed in the B&K Wistar rats (Bkl:WI; Palm et al., 2011b), and the Taconic Han Wistar (HanTac:WH) consumed less alcohol compared to the RCC Han Wistar (RccHan:WI; Palm et al., 2011b; Momeni et al., 2015) with RccHan:WI consuming more alcohol than other Wistar rats of Han origin (Goepfrich et al., 2013). This anomaly of low alcohol intake in B&K Wistar rats, which showed heterogeneity in frequency of the *Grm2* mutation between suppliers (Table 1), unfortunately cannot be confirmed because this source of rats is no longer available.

Adding to the case for a link between alcohol intake and the mutation, alcohol-preferring P but not non-preferring NP rats, selectively bred from a Wistar colony held at the Walter Reed Army Hospital (Lumeng et al., 1977) were homozygous for the cys407* mutation (Zhou et al., 2013). Likewise, the RHA-I but not RLA-I rats

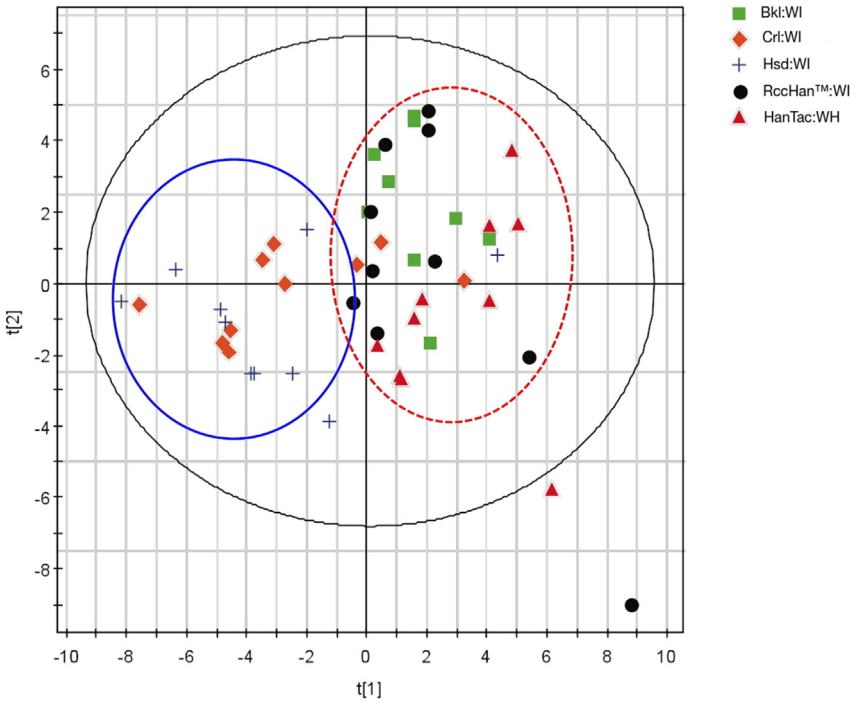

Fig. 3. Principal component analysis (PCA) for individual Wistar rats from five different suppliers behaviourally profiled in the multivariate concentric square field™ test. Modified from Palm et al., (2011a) following the statistical guidelines for PCA analysis of Eriksson et al. (2006). For illustration purposes, the blue line circles the majority (15 of 19) of Wistar rats of non-Han origin, and the red dotted line circles the majority (27 of 29) of Wistar rats of Han origin, i.e. those showing high prevalence for the *Grm2* mutation.

are also homozygous for the cys407* mutation and RHA-I rats have a higher alcohol intake that the RLA-I rats (Manzo et al., 2012; Corda et al., 2014). However, unlike the alcohol-preferring P and RHA-I rats, the Helsinki alcohol-preferring AA (Sommer et al., 2006) have few mutant alleles (Table 2). Therefore segregation between the two lines, and any influence of the *Grm2* mutation on the alcohol drinking characteristic cannot be determined from the AA/ANA lines.

By contrast to the AA/ANA lines, the Warsaw alcohol High-Preferring (WHP) and Low-Preferring (WLP) rats, which were also selectively bred from Wistar stock (see Dyr and Kostowski, 2004), did have a high proportion of cys407* mutant alleles. However, they could not be differentiated with respect to the *Grm2* mutation, with the distribution of the cys407* allele being similar in both WHP and WLP lines (Table 2). But supporting the link between alcohol intake and lack of the mGlu2 receptor, the Sardinian alcohol-Preferring sP and alcohol Non-Preferring sNP rat lines initiated from Wistar stock in 1981 (see Colombo et al., 2006) were clearly distinguished by genotype, the mutant allele being found only in the alcohol-preferring sP line (Table 2).

Thus, despite the anomaly with the B&K and Warsaw rats (see above), these findings strongly support the hypothesis that lack of the mGlu2 receptor contributes to alcohol intake, but is not a requirement (Zhou et al., 2013). Another important pair of selectively bred lines, the high and low alcohol drinking rats (HAD and LAD respectively; Li et al., 1993) have not yet been genotyped. These rats were selectively bred from the N/NIH heterogeneous stock, which in turn were produced by crossing 8 inbred sub-strains, many with some Wistar lineage (Hansen and Spuhler, 1984; Bell et al., 2012), 4 of which were *Grm2* mutants (see Table 3). Interestingly the 4 *Grm2* mutant lines (BUF/N, M520, WN/N and MR/N) were in the top 5 of the 8 N/NIH founders for alcohol preference and consumption (Spuhler and Dietrich, 1984). Our hypothesis above suggests that the mutant cys407* alleles from 4 of the founders will, on selection for alcohol consumption, segregate in the HAD rather than the LAD line. *Note added in revision*: Indeed, HADs have a cys407* mutant allelic frequency of 0.87 versus the 0.5 presumed for the original N/NIH stock (Professor Bill Muir—personal communication).

This hypothesis is also supported by pharmacological data in which mGlu2/3 receptor agonists reduce self-administration in mGlu2-competent rats (Backstrom and Hyytia, 2005) but not in mGlu2-deficient P rats (Rodd et al., 2006). Surprisingly, activation of these receptors did reduce alcohol-seeking behaviour of P rats in the latter study (Rodd et al., 2006). In addition, agonists of mGlu2/3 receptors block the discriminative stimulus effects of alcohol in non-Wistar rats (Cannady et al., 2011) and alcohol seeking behaviour (Besheer et al., 2010). Such data are in agreement with a large body of literature suggesting that mGlu2/3 agonists reduce both the rewarding value of drugs of abuse and the reinstatement of drug seeking behaviour (Moussawi and Kalivas, 2010), most likely because activation of mGlu2/3 receptors reduces dopamine release in the nucleus accumbens shell (Greenslade and Mitchell, 2004; Karasawa et al., 2006). Interestingly, P rats that contain the mutation show a greater alcohol-induced accumbens dopamine response than NP rats whereas there is little difference between AA and ANA rats (Kiianmaa et al., 1995; Bell et al., 2012). Similarly the RHA rats (Manzo et al., 2012) have a larger dopamine response to alcohol than the RLA rats (Corda et al., 2014). These data support the concept that activation of mGlu2 receptors limits the dopamine response to alcohol and other drugs of abuse (see review Moussawi and Kalivas, 2010; Kim et al., 2005; Liechti et al., 2007). Our present data and *inter alia* increased cocaine responsiveness in mGlu2$^{-/-}$ mice (Morishima et al., 2005) and in RHA rats (Giorgi et al., 2007) indicate mGlu2 receptors as a therapeutic target in alcohol and other drug use disorders.

Because anxiety trait and alcohol use disorders have been linked in humans (Morris et al., 2005; Helton and Lohoff, 2015; but see Fein, 2015) and laboratory animals (Stewart et al., 1993; Spanagel et al., 1995; Colombo et al., 1995), it is tempting to ascribe cause and effect to these two aspects of animal behaviour. However, individual rat scores on these two phenotypes within a group of 60 RCC Han Wistar rats were not associated. Instead rats with high risk assessment behaviour displayed higher voluntary alcohol intake (Momeni et al., 2014). Also, against the link between mGlu2 receptor deficit, alcohol preference and anxiety trait is the evidence from the RHA-I and RLA-I rats; the RHA-I rats have the *Grm2* mutation and consume more alcohol but show more aspects of impulsivity, risk-taking and sensation-seeking than the RLA-I rats (Escorihuela et al., 1999; Steimer and Driscoll, 2005; Lopez-Aumatell et al., 2009b). Also, recently the alcohol-preferring P rats (see below), which also have the cys407* mutation (Zhou et al., 2013, Table 2), were found to be more risk-taking than their non-preferring NP and mGlu2 receptor competent counterparts (Roman et al., 2012) in contrast to a previous report suggesting an anxious phenotype for P rats (Stewart et al., 1993). In an attempt to pursue the potential link between the *Grm2* mutation and anxiety (Ceolin et al., 2011), samples from Wistar rats selectively bred for high and low anxiety-related behaviours (HAB and LAB; see Landgraf and Wigger, 2002) were genotyped. Unfortunately for advancing this discussion, all the samples from both HAB and LAB lines were homozygous for the mutation, suggesting that founders came from Wistar stocks with a high percentage of *Grm2* mutants. Similarly, the epileptic GAERS line shows more anxiety-related behaviours than the non-epileptic NEC line (Jones et al., 2008), but does not contain the cys407* mutation.

4.4. Implications for use of cys407* mutant rats in neuroscience research

Although clearly there are large genetic differences between rat strains, the finding that the cys407* mutation of the *Grm2* gene is expressed as the more frequent allele in several commercially available Wistar rat sub-strains is of major concern. The mGlu2 receptor is a key player in many aspects of synaptic transmission and plasticity throughout the CNS (Yokoi et al., 1996; Mukherjee and Manahan-Vaughan, 2013) and is widely regarded as an important target for drug development for neurological and psychiatric diseases (Niswender and Conn, 2010; Nicoletti et al., 2011; Li et al., 2015). Clearly these mutant rats will give atypical results in studies using mGlu2 receptor ligands.

Because many studies have been performed with agonists and antagonists that do not separate between mGlu2 and mGlu3 receptors, both type 1 (an action via mGlu3 receptors may have been ascribed to mGlu2 receptors) and type 2 (lack of effect due to absence of the mGlu2 receptor) errors may have occurred. Published examples are difficult to find because the strains of rats are often not given in sufficient detail and negative results are not always published. It is nevertheless very important that the prevalence of this mutation is recognized by scientists investigating the role of mGlu2 and mGlu3 receptors both physiologically and therapeutically.

By the same token, the cys407* mutation provides researchers with a valuable tool to separate between functions of mGlu2 and mGlu3 receptors, an issue that has slowed drug development in this field (Niswender and Conn, 2010; Nicoletti et al., 2011). As mentioned above, some mGlu2/3 agonists have been withdrawn from further development on the grounds of rat toxicity (Dunayevich et al., 2008), the Han Wistar rats will allow the off-target toxicity to be investigated in isolation. Knock-out mice are available for investigating the influence of individual genes. However, the majority of behavioural tests have been developed in rats, which provide the bulk of rodent behavioural literature (Hanell and Marklund, 2014). Hence, the cys407* mutant rats provide a valuable resource for distinguishing between mGlu2 and mGlu3 receptor functions. Thus the mutant Han Wistar sub-strains provide the opportunity to study the role and therapeutic potential of mGlu3 receptors in the absence of complicating data from interaction of ligands with mGlu2 receptors. For example, the potential for mGlu3 agonism in neuroprotection, although widely appreciated (Bruno et al., 2001; Corti et al., 2007; Caraci et al., 2012; Motolese et al., 2015), has yet to be fully developed and could be more widely explored in these mutant rats.

4.5. General conclusion

Discovery of the cys407* mutation in *Grm2* and its prevalence in Wistar rat sub-strains originating from the Hannover Institute has indicated the likely restraining influence of mGlu2 receptors in animal models of alcohol use disorders in particular, and substance use disorders in general. The data supports the therapeutic opportunity for mGlu2 receptor agonists in research on drug addiction mechanisms and more generally for emotional, risk-related and impulsive behaviours. Rat strains with this cys407* *Grm2* mutation provide a useful model for understanding the separate roles of mGlu2 and mGlu3 receptors in physiology and pathology.

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